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Award Number: W81XWH-11-1-0178

TITLE: Systematic Analysis of the Functional Relevance of Nuclear Structure and Mechanics in Breast Cancer Progression

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REPORT DATE: January 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-01-2012	Annual	1 JUL 2011 - 31 Dec 2011

4. TITLE AND SUBTITLE
Systematic Analysis of the Functional Relevance of Nuclear Structure and Mechanics in Breast Cancer Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0178

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cornell University
Ithaca, NY 14850

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Due to the PI's move from Brigham and Women's Hospital to Cornell University on July 1, 2011, no research was conducted at Brigham and Women's Hospital during the report period (July 1 – Dec 31, 2011). Nonetheless, while waiting for the award transfer to be completed (the official project period start date at Cornell was Feb 1, 2012), we already accomplished several goals of the proposed statement of work. We created panels of cell lines based on two model systems (MCF10A – normal epithelial cells; MDA-MB-231 metastatic breast cancer cells) with systematic alterations in the expression of lamins A, B1, B2, C, and lamin B receptor (LBR). We then evaluated the effect of altered lamin expression on nuclear stiffness in these cell lines. While increased expression of lamin A caused stiffer, less deformable nuclei, reduction of lamins A/C expression by shRNA reduced nuclear stiffness. The effect of alterations in other lamins was less substantial. However, expression of LBR resulted in increased lobulation of the nucleus. These preliminary data support our hypothesis that changes in expression of nuclear envelope proteins recently reported in breast cancer is sufficient to cause altered nuclear morphology and stiffness, which could result in altered migration and invasion.

15. SUBJECT TERMS
Lamins, MCF10A, MDA-MB-231, nuclear stiffness, nuclear shape, lamin B receptor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

The central hypothesis of this proposal is that changes in the expression of nuclear envelope proteins such as lamins or lamin B receptor (LBR) may contribute to the characteristic irregular morphology of cancer cell nuclei and directly modulate cellular functions relevant to cancer progression. Nuclear lamins, particularly lamins A and C, are important determinants of nuclear shape and stiffness.[1-3] At the same time, these proteins also interact with various transcription factors, thereby affecting important signaling pathways.[1] The purpose of this study is to conduct a systematic analysis of the functional consequences of changes in the expression of lamins (A, B1, B2, and C) and lamin B receptor on nuclear morphology and stiffness, as well as the functional consequences of such changes on cell migration through confined spaces (where more deformable nuclei may facilitate enhanced passage), proliferation, and epithelial-to-mesenchymal transition (EMT). In addition, we proposed to conduct an analysis of samples derived from breast cancer patients and orthotopic mouse models of the disease to assess changes in the expression of nuclear envelope proteins in breast cancer samples.

BODY

Change in Institution. The PI's laboratory moved from Brigham and Women's Hospital to Cornell University on July 1, 2011. As a consequence, the award was relinquished by Brigham and Women's Hospital on July 8, 2012 and transferred to Cornell University. The new project period for Cornell University is Feb. 1, 2012 through July 31, 2013. As part of the transfer, the Statement of Work (SOW) was revised to reflect changes in local collaborations. We have gathered new collaborators at Cornell University (Claudia Fischbach, Department of Biomedical Engineering, who will provide tissue samples from an orthotopic mouse model of breast cancer) and the Weill Cornell Medical College in New York City (Linda Vahdat, who will provide tissue samples from breast cancer patients). Despite the move to Cornell University, which entailed the setup of a new laboratory and a delay in the funding for this award (the PI's laboratory moved to Cornell University on July 1, 2011; the transfer of the award was not completed until February 2012), we have already accomplished several of the tasks outline in the SOW. Please note that since the project period covered in this report is July 1 – December 31, 2011, most of the work reported here was already conducted at Cornell University.

***Task 1:* Acquire a panel of cell lines and patient-derived samples representing various stages of breast cancer progression from benign to metastatic (Months 0–18)**

We have focused our initial efforts on a subset of cell lines to optimize experimental techniques. We have obtained the following cell lines: MCF10A (normal mammary epithelial cells), MDA-MB-231 (metastatic breast cancer cells), and MCF7 (non-metastatic breast cancer cells). To obtain patient samples, we have established a collaboration with Linda Vahdat at Weill Cornell Medical College, who will provide paraffin embedded tissue sections that we can use to assess expression levels of nuclear envelope proteins by immunofluorescence and immunohistochemistry. Since the Weill Cornell Medical College does not have any RNA samples readily available, we are still exploring different options to obtain DNA and RNA samples from patients, including the use of commercial and academic tissue banks, as well as from the Dana-Farber Cancer Center, as initially proposed. The only complication thus far has been that the contacts at the Dana-Farber Cancer Center have been less responsive after the PI's move to a new institution. Regarding the collection of tissue sections from a breast cancer mouse model, we are working with Claudia Fischbach's laboratory at Cornell University, who has already provided us with samples to optimize our staining procedures.

***Task 2:* Modulate nuclear shape and stiffness in a panel of well characterized breast cancer cells and non-tumorigenic controls by stable, ectopic expression of lamins A, B1, B2, C, a dominant negative lamin A mutant, or lamin B receptor (LBR) (Months 1–18)**

We have created a panel of cell lines derived from MCF10A and MDA-MB-231 cells in which we selectively overexpressed lamin A, lamin B1, lamin B2, lamin C, or LBR with a custom-designed retroviral construct followed by fluorescence activated cell sorting to obtain physiological expression levels. In addition, we created a corresponding panel of cell lines in which we reduced expression of lamins A/C, lamin B1, lamin B2, and LBR by shRNA mediated knockdown. We confirmed changes in protein levels by Western blot analysis (Fig. 1). Overall, the modulation of nuclear envelope protein expression worked very well, particularly for lamins A/C and LBR. For B-type lamins, we experienced some difficulties obtaining stable cell lines with very high or very low expression levels, which speak to the importance of these proteins in maintaining normal cell function. We will continue to work with the cell lines with moderate changes in B-type lamin protein expression, but we will also include studies on cells with transiently modified protein expression, which might enable us to achieve more severe changes in expression compared to stable cell lines.

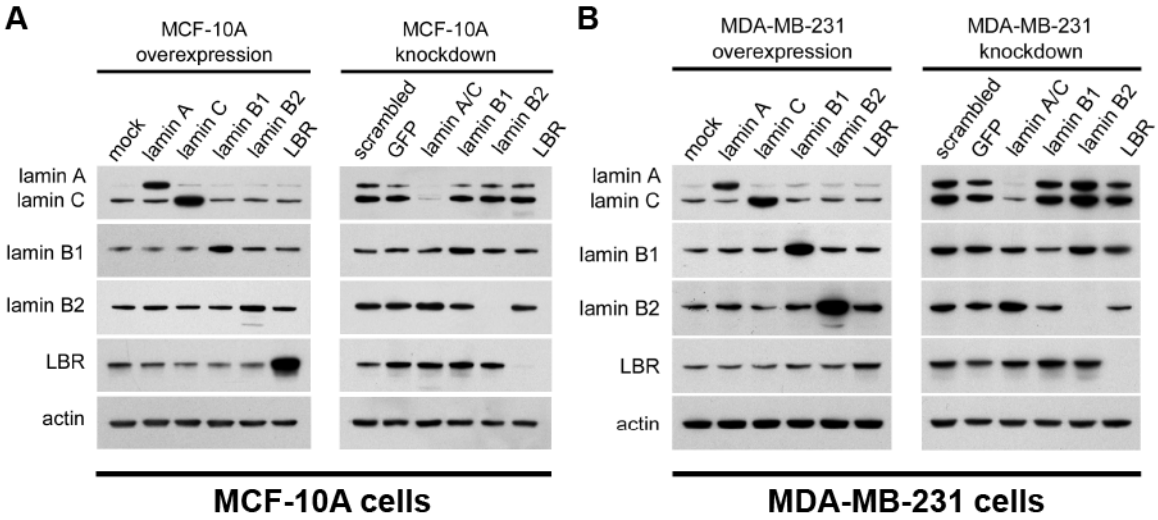

Figure 1. Western analysis of cells modified to express different levels of nuclear envelope proteins. Overexpression was achieved with the bicistronic retroviral constructs and subsequent fluorescence activated cell sorting (FACS). Knockdown of specific nuclear envelop proteins was achieved by shRNA lentiviral constructs and subsequent selection. (A) Results for MCF10A normal breast epithelial cells. (B) Results for MDA-MB-231 breast cancer cells.

We next evaluated the effect of changes in protein expression on nuclear morphology (Fig. 2). We found that most modifications had relatively minor effects on nuclear shape. However, overexpression of LBR resulted in nuclei with severe lobulations (Fig. 2), whereas overexpression of lamin A resulted in rounder nuclei. These results were confirmed by quantitative analysis of nuclear 'roundness' using the Contour Index (CI), defined as

$$CI = 4\pi \times (\textit{nuclear cross-sectional area}) \,/\, (\textit{nuclear periphery})^2$$

The *CI* reaches a maximum of 1 for perfectly circular nuclei and decreases with increasingly irregular nuclear shape. Knockdown of lamins A/C and of lamin B2 resulted in more irregularly shaped nuclei, indicated by a decrease in the corresponding *CI* (Figs, 2 & 3). In contrast,

overexpression of lamin A resulted in rounder nuclear with an increased *CI*. Overexpression of LBR caused severe lobulation of nuclei in MCF10A cells, resembling those of cancer cells (Fig. 2).

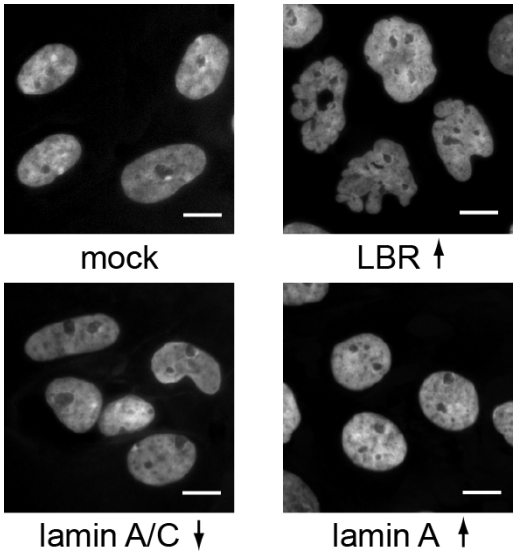

Figure 2. Examples of altered nuclear morphology in MCF10A cells with altered expression of lamin A or LBR, revealing increased nuclear lobulation in cells with reduced expression of lamin A/C or increased expression of LBR. Cells overexpressing lamin A have rounder nuclei.

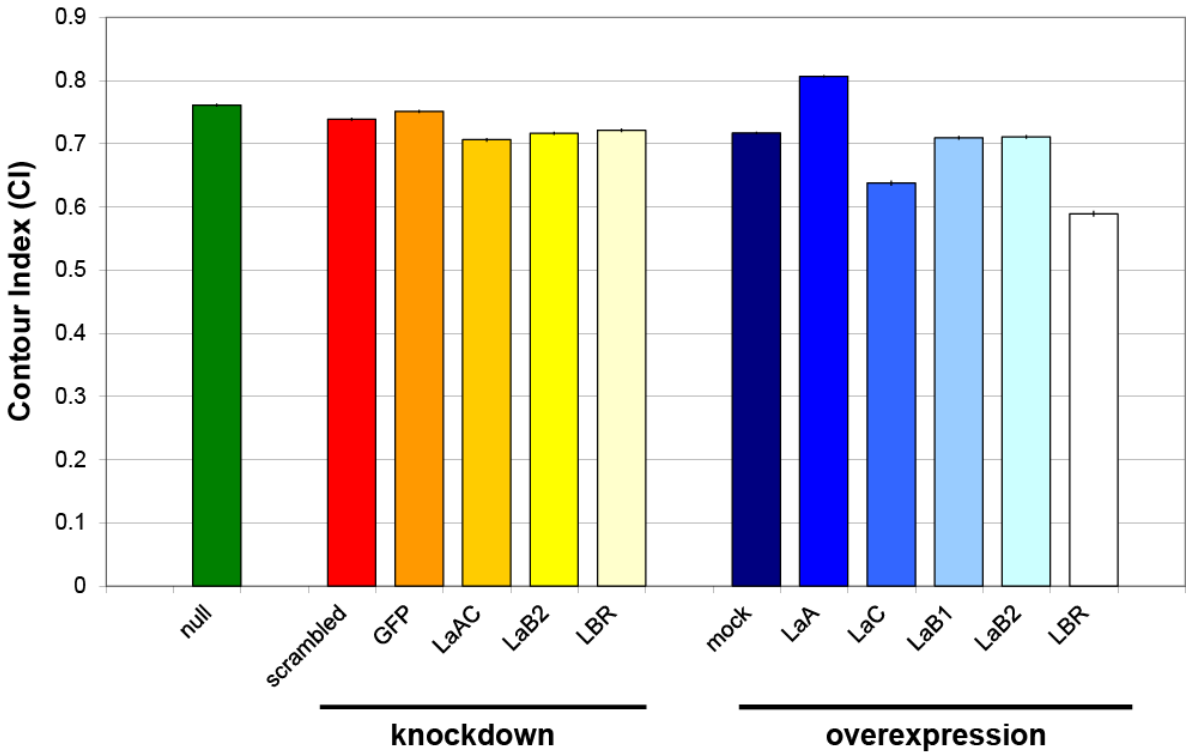

Figure 3. Quantitative analysis of nuclear shape in MCF10A cells with altered expression of nuclear envelope proteins. Data based on ~500 to 1000 cells per cell lines. Null, non-modified controls.

To assess the effect of altered nuclear envelope protein expression on nuclear deformability, we subjected the panel of modified MCF10A cells to substrate strain experiments and measured the resulting nuclear deformations. The normalized nuclear strain, defined as the induced nuclear strain divided by the applied substrate strain, can serve as a direct indicator for nuclear stiffness.[3, 4] Lower normalized nuclear strain values indicate stiffer nuclei, while higher values, corresponding to larger nuclear deformations, indicate softer nuclei. In our experiments, we found that increased expression of lamin A significantly increased nuclear stiffness, while reduced expression of lamins A/C dramatically decreased nuclear stiffness (Fig. 4). The effects of changes in the expression of other nuclear envelope proteins were less dramatic, but still significant, particularly for overexpression of lamin C, lamin B1, and LBR, which reduced nuclear stiffness.

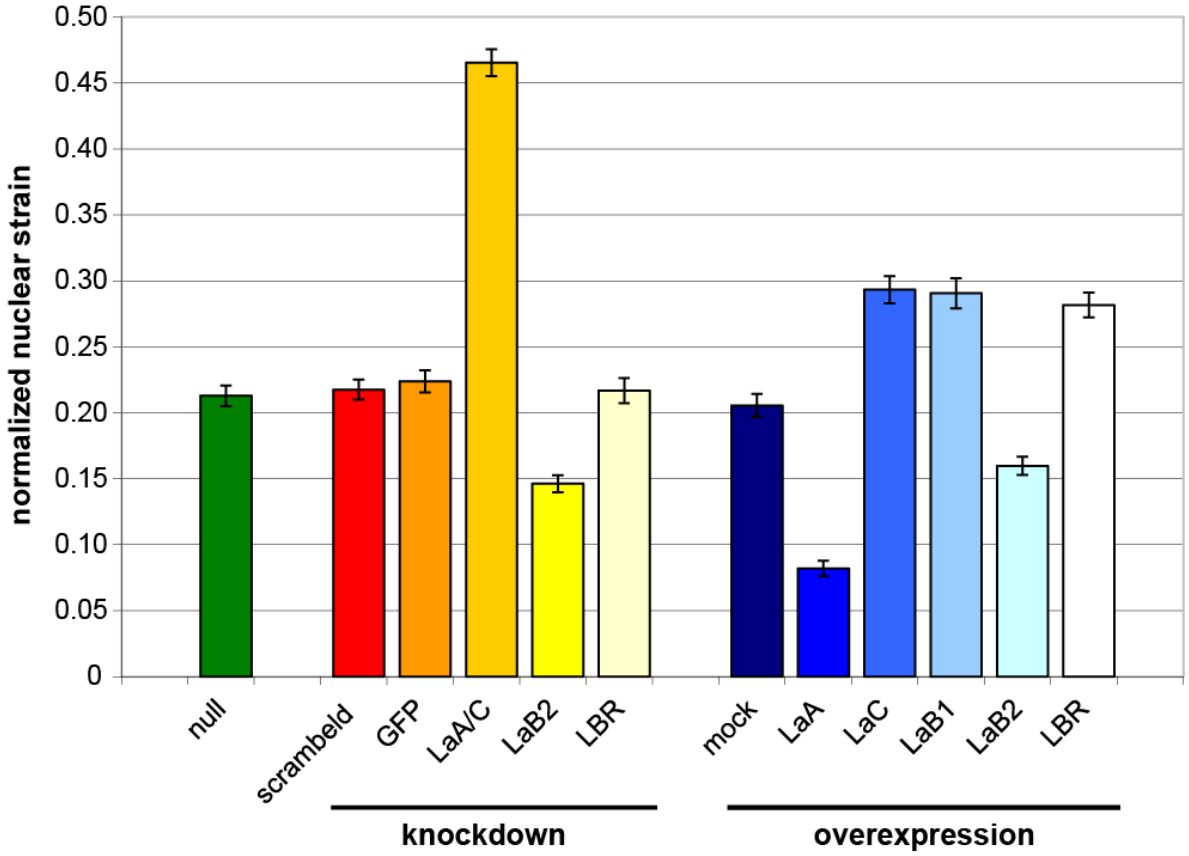

Figure 4. Effect of altered expression of nuclear envelope proteins on nuclear deformability. Lower normalized nuclear strain values indicate stiffer, less deformable nuclei, while higher value indicate softer nuclei.

<u>Task 3:</u> Investigate whether changes in nuclear shape or stiffness can alter invasion, migration, or perfusion through narrow channels in the newly created panel of cell lines (Months 6–24)

We have already begun with the preparations for this task, designing microfluidic devices for the perfusion and migration of breast cancer cells. We are currently in the process of further optimizing the devices and the experimental setup for higher throughout studies.

***Task 4:* Quantify expression levels of lamins and LBR in breast cancer cell lines, patient-derived breast cancer cells/tissue sections and determine correlation with disease progression from benign to more aggressive/metastatic phenotypes (Months 1–24)**

We have already analyzed expression of lamins and LBR in MCF10A, MDA-MB-231, and MCF7 cells. We have also begun with the immunohistochemical analysis of lamin expression in tissue sections. We encountered some delays in the process due to *(i)* availability of samples and *(ii)* problems with non-specific staining. We have addressed the first issue by collaboration with Linda Vahdat, who will provide us with a larger number of breast cancer patient samples. If necessary, we can supplement these samples with samples obtained from commercial and academic tissue banks, including the Ontario Tumour Bank (http://oicr.on.ca/oicr-programs-and-platforms/technology-platforms/ontario-tumour-bank) or Asterand (http://solutions.asterand.com/default.asp). In addition, we will take advantage of publically available results analysis of previous tumor expression profiling studies, including the public gene expression data sets from NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/), which includes several data sets for breast cancer tissue samples and breast cancer lines as listed below. We will analyze these data sets for changes in expression of lamins and LBR and possible correlations between changes in expression and disease severity or progression. Regarding the second issue with non-specific staining, we have spent a substantial amount of time optimizing the staining procedures, testing various antibodies and different processing steps, resulting in promising results.

Data Set	Description
GDS2635	Invasive ductal and lobular breast carcinomas
GDS2617	Tumorigenic breast cancer cells (HG-U133A)
GDS2618	Tumorigenic breast cancer cells (HG-U133B)
GDS2250	Basal-like breast cancer tumors
GDS2045	Ductal carcinoma in situ to invasive ductal carcinoma progression (HG-U133A)
GDS2046	Ductal carcinoma in situ to invasive ductal carcinoma progression (HG-U133 2.0)
GDS817	Breast cancer cell expression profiles (HG-U95A)
GDS820	Breast cancer cell expression profiles (HG-U133A)
GDS823	Breast cancer cell expression profiles (HG-U133B)
GDS825	Breast cancer cell expression profiles (G4100A)
GDS88	Cancer cell lines (10k_print2)

Table 1. Overview of publically available data sets through NCBI's GEO database for gene expression profiles relevant to breast cancer.

Our hypothesis that alterations in lamin expression could contribute to breast cancer progression is further supported by a recent report in the *Chinese Journal of Cancer*, which described that lamins A/C are absent in almost 40% of human breast cancer tissues and that even in lamin A/C-positive cancers, expression of lamin A/C is heterogeneous or marked by altered intracellular distribution in the tumor cells.[5] The same report also indicated that in most breast cancer cell lines have a significant fraction of lamin A/C-negative cells.[5]

Taken together, these findings suggest that downregulation of lamins A/C is prevalent in human breast cancers; the resulting changes in nuclear morphology and stiffness could then result in enhanced migration of cells through narrow constrictions, which we are currently evaluating.

KEY RESEARCH ACCOMPLISHMENTS

- Creation of cell panel based on MCF10A and MDA-MB-231 cell lines with systematic variation in the expression of the nuclear envelope proteins lamin A, lamin B1, lamin B2, lamin C, and lamin B receptor (LBR) by shRNA mediated knockdown or ectopic expression
- Confirmation of changes in levels of targeted proteins by Western blot analysis
- Characterization of the effect of changes in nuclear envelope composition on nuclear deformability – increased expression of lamin A caused the most severe increase in nuclear stiffness; decreased expression of lamins A/C resulted in the most severe decrease in nuclear stiffness
- Characterization of the effect of altered expression of nuclear envelope proteins on nuclear shape – increased expression of LBR resulted in more lobulated nuclei. Changes in the expression of other nuclear envelope proteins had milder, but still significant effects.
- Established new collaboration with Linda Vahdat at Weill Cornell Medical College to obtain samples from breast cancer patients and started optimization of immunohistochemistry and immofluorescence procedures
- Established new collaboration with Dr. Claudia Fischbach's laboratory in the Department of Biomedical Engineering at Cornell University, who has extensive experience with orthotopic breast cancer models in mice models and who has already provided us with tissue sections from these mouse models.

REPORTABLE OUTCOMES

Manuscripts

We published the following manuscripts acknowledging funding from this project. Please note that these articles appeared in 2012, i.e., after the end of the report period (Jul – Dec 2011).

1. Ho CY, **Lammerding J**. (2012). Lamins at a Glance. *J Cell Sci*. 125 (9): 2087-2093.
2. Isermann P, Davidson PM, Sliz JD, **Lammerding J**. (2012). Assays to measure nuclear mechanics in interphase cells. *Current Protocols in Cell Biology*. In press.

Cell lines

We created the following cell lines:

- MDA-MB-231 cells overexpressing either lamin A, lamin B1, lamin B2, lamin C or lamin B receptor (LBR)
- MDA-MB-231 with reduced expression of either lamins A/C, lamin B1, lamin B2, or LBR
- MCF10A cells overexpressing either lamin A, lamin B1, lamin B2, lamin C or LBR
- MCF10A with reduced expression of either lamins A/C, lamin B1, lamin B2, or LBR

Conferences/Seminars/Meetings

Work performed as part of this project has been/will be presented at the following meetings:

- "Intracellular Mechanics and Mechanosensing in Physiology and Disease". Invited seminar at the College of Engineering, Montana State University, Bozeman, MT in April 2012.
- Breast Cancer Research Retreat of the Weill Cornell Medical College in New York, NY, in Oct. 2012
- Physics of Cancer 2012 Symposium at the University of Leipzig, Germany, in Nov. 2012

- "Exploring nuclear deformability as a rate-limiting factor in cancer cell migration", selected for a platform presentation at the 2012 Annual Meeting of the Biomedical Engineering Society (BMES) in Atlanta, GA, in Oct. 2012

CONCLUSION

Our data acquired so far demonstrate that loss of lamins A/C, which frequently occurs in human breast cancers, results in more lobulated and severely more deformable nuclei. A similar effect was observed as a result of overexpression of LBR. Increased deformability of the normally large and stiff nucleus could aid cells in the translocation through narrow constrictions, whether it is during perfusion through capillaries, intra- or extravasation, or migration through dense tissues. The research, once completed, could have important clinical implications. Analysis of expression levels of nuclear envelope proteins could be used in the diagnosis and particularly the prognosis of breast cancers, where a high fraction of cells with softer nuclei could indicate higher risk to the patient. Such prognostic approaches would be particularly powerful when applied to the analysis of circulating tumor cells, as it may help identify particularly aggressive subpopulations of tumor cells.

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APPENDICES

None

SUPPORTING DATA

None – all figures are included in the text above